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Award Number: DAMD17-01-1-0240

TITLE: Investigation of the Link Between Prolactin, Mammary Gland Development and Carcinogenesis by Transcript Profiling

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REPORT DATE: October 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2002	Annual Summary (1 Oct 01 - 30 Sep 02)

4. TITLE AND SUBTITLE
Investigation of the Link Between Prolactin, Mammary Gland Development and Carcinogenesis by Transcript Profiling

5. FUNDING NUMBERS
DAMD17-01-1-0240

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words) (abstract should contain no proprietary or confidential information)*

Mammary lobuloalveolar development during pregnancy is controlled by the endocrine action of prolactin, estrogen and progesterone, but currently little is known of the transcriptional events that occur in response to these hormones. This study examines the transcriptional response of the mammary gland to the direct action of prolactin. Mammary tissue recombination experiments demonstrated that lobuloalveolar development is dependent upon the presence of prolactin receptors in mammary epithelium but not the stroma. Comparison of transcript profiles derived from recombined glands with and without epithelial prolactin receptors, from glands without epithelium, and from SCp2 cells treated with and without prolactin, allowed a small set of epithelial-specific prolactin-modulated transcripts to be identified. This included the epithelial specific Ets transcription factor Elf5. Elf5 expression decreased in $Prlr^{+/-}$ mammary glands but there was no change in Prlr expression in $Elf5^{+/-}$ mammary glands, indicating that Elf5 acts downstream of prolactin. The expression of milk protein genes decreased in both $Prlr^{+/-}$ and $Elf5^{+/-}$ mammary glands, consistent with a role for Elf5 in mediating prolactin-induced epithelial differentiation, however, the expression of a number of growth factors decreased in $Prlr^{+/-}$ mammary glands but increased in $Elf5^{+/-}$ mammary glands. Thus prolactin induces the expression of Elf5, which performs a pivotal role in mediating the switch from prolactin induced epithelial cell proliferation to differentiation within the mammary gland during pregnancy.

14. SUBJECT TERMS:
breast cancer, prolactin, mammary gland development

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Mouse mammary gland development is initiated at mid gestation and by birth a few short mammary ducts have formed. These undergo simple allometric growth prior to puberty after which the ducts elongate and bifurcate to fill the mammary fat pad, their density increasing with each estrous cycle. During pregnancy further side branching occurs and cell proliferation and differentiation within the alveolar buds at the ductal termini results in the formation of large lobuloalveolar structures, facilitating milk production and secretion. Following parturition, full functional differentiation occurs and lactation commences. At weaning the gland commences involution, with the loss of most of the epithelial component gained during the preceding lactation (1).

The initiating factors for the developmental events during puberty and pregnancy are endocrine, mediated by increases in the levels of circulating ovarian estrogen and progesterone, which act in concert with increased pituitary prolactin secretion.

Loss of the prolactin receptor (Prlr) in the mouse mammary gland (2, 3), prevents side branching following puberty, but indirectly via loss of progesterone secretion from the corpora lutea (4). During pregnancy, loss of Prlr prevents the cell proliferation required to build the lobuloalveoli, and development stops at a stage following the formation of alveolar buds (2). In contrast, loss of a single Prlr allele has no effect on cell proliferation and development proceeds past the formation of alveolar buds to produce lobuloalveolar structures with normal architecture (2), but these structures fail to fully differentiate and lactation fails (3). How prolactin can initiate and control the competing processes of cell proliferation during early pregnancy and functional differentiation during late pregnancy is unknown.

This project aims to elucidate the transcriptional cascade that begins with alterations in the level of prolactin action and ends with development of the lobuloalveoli and lactation. We aimed to identify genes important for the proliferation and differentiation mediated by prolactin in the mammary gland during pregnancy and to asses these genes for relevance to breast cancer.

Key Research Accomplishments

- *Task1.* Preparation of Mammary Glands for Transcript Profiling and
- *Task2.* Transcript Profiling

Mammary epithelium from $Prlr^{+/+}$ and $Prlr^{-/-}$ animals was transplanted into the cleared fat pads of 4 to 6 $Rag1^{-/-}$ animals. The epithelium was allowed to develop under the normal endocrine environment of the host animals for 12 weeks upon which the animals were time-mated and the glands collected at 2, 4 and 6 days of pregnancy. Glands without epithelium were prepared in the same way. Quality RNA was prepared from each gland and pooled for labeling according to Affymetrix protocol. The labeled RNA was hybridised to MGU74A Affymetrix GeneChips and data accumulated using Microarray Suite (MAS) 4.0. Problems with the design of the MGU74 GeneChips led to the data being re-analysed using a probe mask to filter out the incorrectly designed probes.

2 *Task3* Gene Expression Analysis

A number of methods were employed to analyse the large amount of data generated by these experiments. Heirarchical clustering was used to establish the relationship between each of the glands profiled. This analysis confirmed that we could detect differences in the mammary transplants purely at the level of gene expression (Figure One).

The Affymetrix Data Mining Tool was used to generate a spreadsheet containing all the data and this was manipulated in Excel to identify potential candidates for further study. These candidates were selected by identifying genes that decreased in the $Prlr^{-/-}$ mammary transplants when compared to the $Prlr^{+/+}$ mammary transplants and genes that were not found in the fat pad cleared of epithelium in at least 2 of the three days of early pregnancy profiled. Over 200 genes modulated by prolactins action on the mammary epithelium were identified in this way. To enable us to visualise the data Principal Components Analysis (PCA) was performed on log transformed data and the genes of interest labeled according to the MAS 4.0 calls (Figure Two). Differences seen in fifteen genes known to be important for mammary development was confirmed by quantitative PCR.

Transcript profiling of mammary SCp2 cells treated with prolactin was also performed. Although originally not in the statement of work these experiments were necessary to reduce the number of

candidates to a few interesting genes for further analysis. These experiments contrasted with the transplant experiements which are a model of loss of prolactin action. SCp2 mammary epithelial cells when grown on matrigel and treated with prolactin form mammospheres and express β-casein, a known prolactin regulated milk protein gene (5). We transcript profiled this model of positive prolactin action and compared cells treated with prolactin and without prolactin to the transplant experiments. This gave us a much smaller list of genes from which to select our candidates for further investigation (Table One).

- *Task 4.* Tissue Arrays

Two genes were chosen for further investigation – an ETS transcription factor, Elf5 and an Expressed Sequence Tag (EST) of unkown function. Through collaboration with Dr Melanie Pritchards group in Melbourne we were able to establish that the $Elf5^{+/-}$ mouse has the same mammary phenotype as the $Prlr^{+/-}$ mouse – that of reduced lobuloalveolar development . Like the $Prlr^{+/-}$ phenotyope the Elf5 defect is specific to the mammary epithelium. To asses the similarities and differences between the $Prlr^{+/-}$ and the $Elf5^{+/-}$ mammary galnds we looked at the level of expression of a number of genes identified the transcript profiling experiments known to be imporatant for mammary development. The expression of milk protein genes decreased in both $Prlr^{+/-}$ and $Elf5^{+/-}$ mammary glands, consistent with a role for Elf5 in mediating prolactin-induced epithelial differentiation, however, the expression of a number of growth factors decreased in $Prlr^{+/-}$ mammary glands but increased in $Elf5^{+/-}$ mammary glands. Thus prolactin induces the expression of Elf5, which performs a pivotal role in mediating the switch from prolactin induced epithelial cell proliferation to differentiation within the mammary gland during pregnancy. Elf5 may play a vital tumor-suppressor role in the mammary gland.

ELF5 is located in a region of the human genome known to experience loss of heterozygousity in breast cancer (6). ELF5 expression in a panel of breast cancer cell lines was performed by quantitative PCR, and suggests that ELF5 expression may be linked to the hormone receptor status of the cell line.

Extensive database searching was performed to identify the gene associated with the candidate EST – to date both the mouse gene and its human orthologue have been identified.

Tissue arrays of breast cancer samples have been constructed, althought there was some delay in

completion of this task due to the large amount of data acquisition required to document this process. The protocol for making *in situ* probes for the EST has been optimised and an antibody for Elf5 obtained. Optimistion of the staining protocols for identification of these molecules in breast tissue is currently being undertaken and it is expected that staining of the entire breast cancer cohort will occur in early 2003.

- *Task 5*. Final Analyses and Report Writing

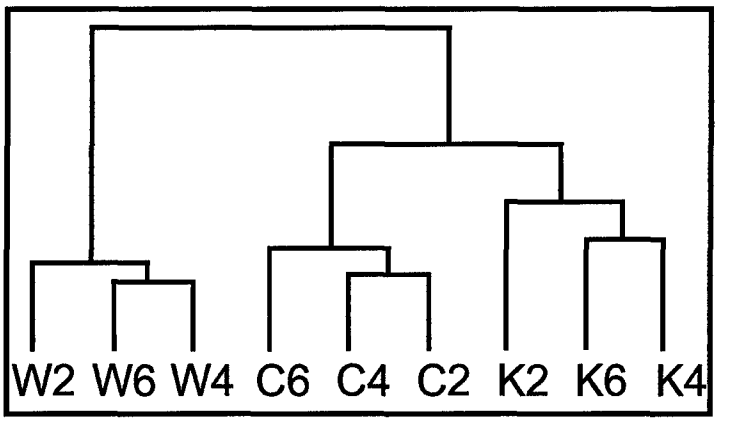

Figure One: Hierarchical Clustering of Transplanted Mammary Glands, W: Prlr+/+ epithelial transpl;ants, C: Prlr+/+ fat pads cleared of epithelium, K: Prlr-/- epithelial transplants

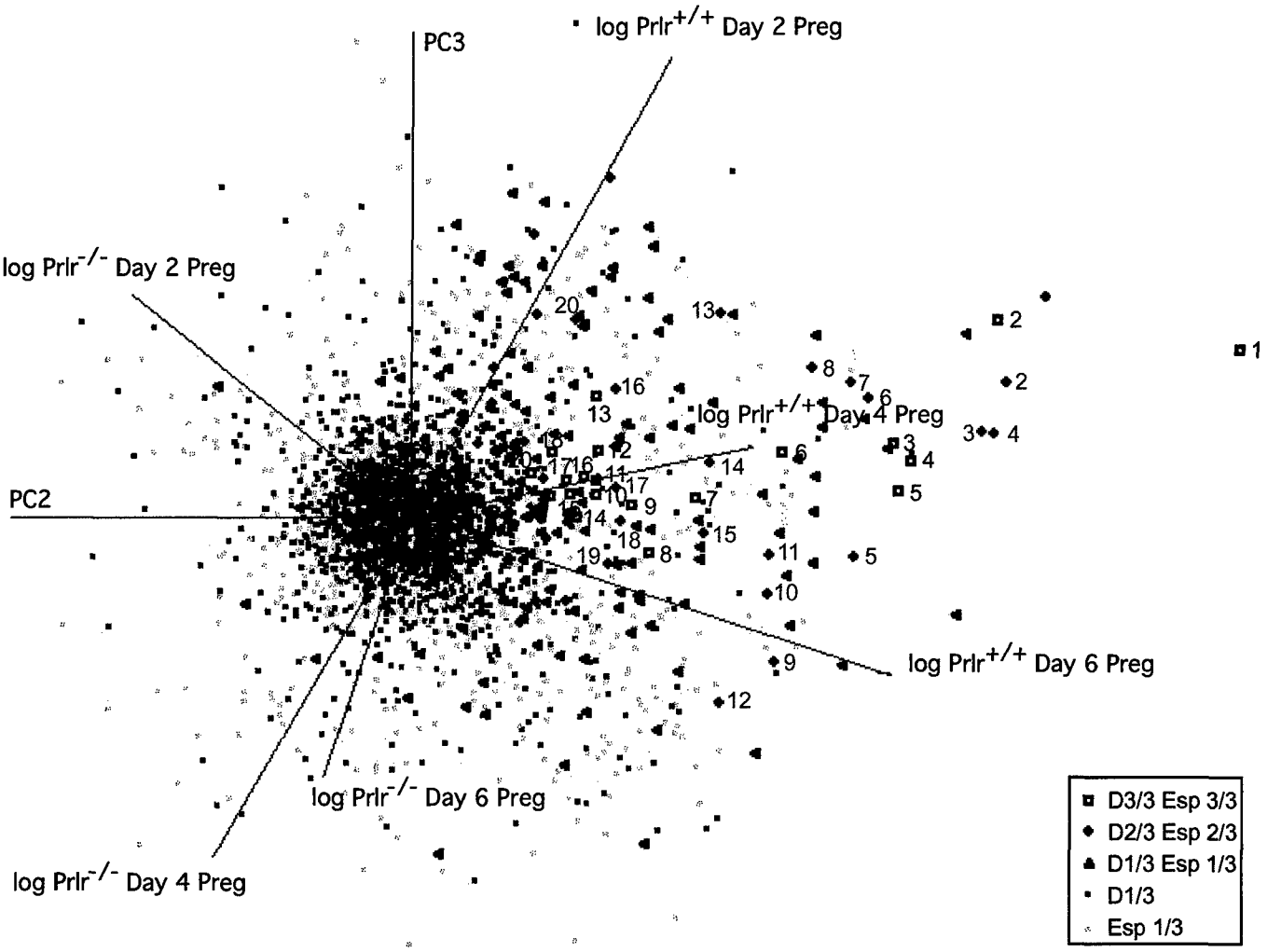

Figure Two. Principal component analysis of Prlr+/+ and Prlr-/- epithelial transplants at day 2, 4 and 6 of pregnancy, using log2 of the average difference values from MAS 4.0. The second principal component (x) graphed verses the third principal component (y). The first principal component (z) runs through the page. Genes were labeled according to MAS 4.0 calls: Red Squares, decreasing in Prlr-/- transplants at all three days (D3/3) and epithelial at all three days (Ep3/3). Purple Diamonds, decreasing in Prlr-/- transplants at any two of three days (D2/3) and epithelial at any two of three days (Ep2/3). Blue Triangles, decreasing in Prlr-/- transplants at any one of three days (D1/3) and epithelial at any one of the three days (Ep1/3). Green Squares, decreasing in Prlr-/- transplants at any one of the days (D1/3). Yellow Squares, epithelial at any one of the three days (Ep1/3).

Genbank	Name	Symbol	Annotation	day2	day4	day6	SCp2 E1	SCp2E2
M38724	cell division cycle 2A	Cdc2a	cell cycle	-1.8	-2.8	-3.6	-1.2	2
X93037	WDNM1	Expi	proteinase inhibitor	-10.1	-9.5	-8.7	2.2	2.1
AF049702	E74-like factor 5	Elf5	transcription factor	-2.6	-2.5	-3.5	1.8	A
X60367	retinol binding protein 1	Rbp1	transport	-4.1	-3.8	-4.3	1.3	1.3
U06119	cathepsin H	Ctsh	proteolysis and peptidolysis	-1.9	-1.8	-1.7	-1.2	1.3
AI838452	v-ral	Rala	GTPase	-1.9	-2.3	-1.3	2.8	A
AI851740	actin related protein	Arpc3	cytoskeleton	-1.8	-1.4	-1.8	1.8	-1.1
AW049647	ADP-ribosylation 6, 5	Arl6ip5	intracellular trafficking	-2.4	-2	-2.1	1.8	-1.2
AA693246	U2	U2af1	mRNA splicing	-2.4	-1.8	-2.5	1.8	1.1
M10114	casein kappa	Csnk	milk protein	-4.1	-5	-2.9	3.6	2.6
X04490	casein beta	Csnb	milk protein	-1.2	-6.7	-11.0	14.6	19.7
M36780	casein alpha	Csna	milk protein	-5.6	-15.8	-41.0	5.2	2.5
U88327	SOCS2	Cish2	cytokine signaling	-1.9	-1.7	-1.7	2.1	1.8

Table One: Candidate genes (does not include ESTs) identified be transcript profiling mammary transplants at days two, four and six of pregnancy and SCp2 cells treated with prolactin.

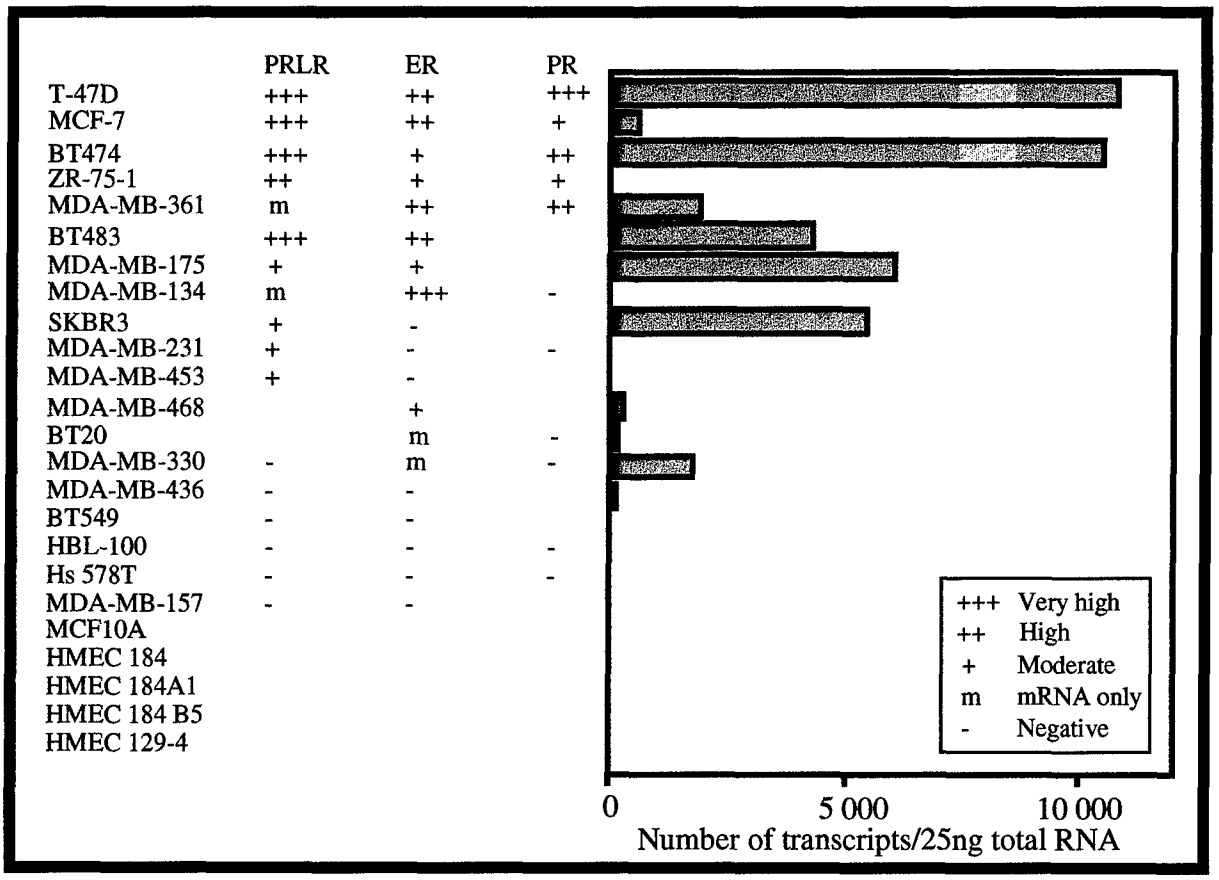

Figure Three: ELF5 expression in breast cancer cell lines and their hormone receptor status.

Reportable Outcomes

- **Manuscripts**

Investigation of the transcriptional changes underlying functional defects in the mammary glands of prolactin receptor knockout mice.

Christopher J. Ormandy, Matthew Naylor, Jessica Harris, Fiona Robertson, Nelson D. Horseman Geoffrey J. Lindeman, Jane Visvader and Paul A. Kelly

Recent Progress in Hormone Research, *in press*

Elf-5 an Ets transcription factor is a key modulator of prolactin action on the mammary epithelium.

Jessica Harris, Matthew J. Naylor, Fiona G. Robertson, Sergio Wittlin, Jiong Zhou, Erika J. Lapinskas, Paul J. Hertzog, Geoffrey J, Lindeman, Jane Visvader, Paul A. Kelly, Melanie A. Pritchard and Christopher J. Ormandy

Journal of Clinical Investigation, *submitted*

The mouse Elf5 gene is essential for early embryogenesis and mammary gland development during pregnancy and lactation.

Jiong Zhou, Trevor J Wilson, Erika J. Lapinskas, Matthew J. Naylor, Jessica Harris, Sue Tsao, Silva Zavarsek, Dakang Xu, Jane Visvader, Geoffrey J, Lindeman, Ross Thomas, Christopher J. Ormandy Paul J. Hertzog, Melanie A. Pritchard and Ismail Kola.

Journal of Clinical Investigation, *submitted*

Local insulin-like growth factor-II mediates prolactin-induced mammary gland development.

Russell C. Hovey, Jessica Harris, Darryl L. Hadsell, Adrian V. Lee, Christopher J. Ormandy and Barbara K. Vonderhaar

Molecular Endocrinology, *in press*

Presentations

U.S Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope Meeting

Orlanda, Florida, September 2002

"Transcript profiling of prolactin-dependent models of mammary development reveals genes important for lobuloalveolar development"

2nd Australian Microarray Meeting

Stradbroke Island, Queensland, July 2002

"Transcript profiling of prolactin-dependent models of mammary development reveals novel genes involved in the prolactin response in the mammary gland during pregnancy"

22nd Annual Conference on the Organisation and Expression of the Genome

Lorne, Australia, February 2001

"Transcript profiling of mammary epithelial transplants from prolactin receptor knockout and wild-type mice using the Affymetrix system"

- **Abstracts**

2002 Gordon Research Conference on Prolactin

Ventura, California, January 2002

"Identification of genes involved in the prolactin response in the mammary gland during pregnancy by transcript profiling of epithelial transplants from prolactin receptor knockout and wild-type mice"

Conclusions

The pituitary hormone prolactin acts directly on the epithelium to promote lobuloalveolar development in the mammary gland during pregnancy. We have combined the techniques of transcript profiling with mammary epithelial transplants and cell culture to identify the genes active within the epithelium and responsible for mediating the effect of prolactin on the mammary gland. Many of the genes identified have established roles in mammary gland development, providing confirmation of the discriminatory power of our model. We have identified Elf5 as a transcription factor acting in the mammary epithelium to promote lobuloalveolar development and have chosen this gene and one novel gene for further analysis in breast cancers.

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